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TESTING OF THE BIO-SEEQ® (SMITHS DETECTION HANDHELD PCR INSTRUMENT): SENSITIVITY, SPECIFICITY, AND EFFECT OF INTERFERENTS ON *YERSINIA PESTIS* ASSAY PERFORMANCE

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14. ABSTRACT
Smiths Detection-Edgewood (SDE), Inc., recently developed a handheld PCR instrument, the Bio-Seeq®, an updated, redesigned version of a small, portable PCR instrument previously known as Handheld Advanced Nucleic Acid Analyzer (HANAA). The SDE developed the Bio-Seeq® to provide a portable platform for use by first responders to detect biological threats in civilian areas. To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. In previous work, SDE obtained probe and primer sequences constituting an assay for a gene present in *Yersinia pestis,* the causative agent of plague. The SDE incorporated this probe and primer set into dried reagent beads, which also contain reagents required for an internal control. These, in turn, are part of a self-contained sampling device that contains buffer and the PCR reagent beads. This report details an analysis of the assay for *Y. pestis*, including the sensitivity and specificity of the assay, and the effect of some common non-target (interferent) materials on the performance of the assay.

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PREFACE

The work described in this report was authorized under the Cooperative Research and Development Agreement Project No. 0309C, between the U.S. Army Edgewood Chemical Biological Center and Smiths Detection-Edgewood. This work was started in December 2003 and was completed in June 2004.

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1. INTRODUCTION AND BACKGROUND

In May 2003, the Edgewood Chemical Biological Center (ECBC) and Smiths Detection-Edgewood (SDE), Inc. entered into a Cooperative Research and Development Agreement (CRADA Project 0309C) for the purpose of developing assays for SDE's handheld PCR instrument, the Bio-Seeq®. The SDE developed the Bio-Seeq® to provide a portable platform for use by first responders to detect biological threats in civilian areas. The Bio-Seeq® is an updated, redesigned version of small, portable PCR instrument previously known as Handheld Advanced Nucleic Acid Analyzer (HANAA).[1,2] At least one reference to its use in detecting a BW agent exists in the peer-reviewed literature.[3] Information on the Bio-Seeq® is available from the manufacturer at http://194.105.117.18/products/Default.asp?Product=6&section=Military.

To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. Assays that are designed for any instrument that can accommodate real-time fluorogenic PCR can most likely be adapted for use with the Bio-Seeq®. Molecular biologists at ECBC have experience in the development of real-time fluorogenic PCR assays ("TaqMan") for pathogen detection.

In previous work, SDE obtained probe and primer sequences constituting an assay for a gene present in cells of *Yersinia pestis*, the causative agent of plague.[4] The SDE incorporated this probe and primer set into dried reagent beads, which also contain reagents required for an internal control. These in turn are part of a self-contained sampling device that contains buffer and the PCR reagent beads.

2. OBJECTIVE

The objective of the work reported here was to answer the following questions:

a. Is the assay (the probe and primer set) specific for *Yersinia pestis*?

b. How sensitive is the assay when target cells are applied directly to the consumable sampler?

c. What is the effect of some common non-target (interferent) materials on the performance of the assay when target cells are used?

3. SCOPE

This study is the third part of an overall plan to test the sensitivity, specificity, and resistance to interferents of three assays designed for use in the Bio-Seeq® instrument. The probe and primer sets in the assays are designed to each detect one of the following threat agents: *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*.

4. METHODS AND MATERIALS

4.1 Instrument Description.

Smiths Detection Edgewood, Inc. is the manufacturer of the Bio-Seeq® instrument (Figure 1). Three Bio-Seeq® units (s/n 115, 122, and another unit dubbed "thermal paste") were provided to ECBC by SDE. Each unit contains six thermocycler modules that are independently programmable and operable. Each module can be run separately. The instrument has an LCD panel that allows an operator to enter, store, and run programs that instruct each module to heat and cool a PCR reaction tube to a specified temperature for a specified length of time, within the operating parameters of the instrument. A user can also monitor and gather data from each module independently using a PC that is running proprietary software developed by SDE (latest version at this writing is 1.21). When connected to a PC, the instrument will graphically display the development of the fluorescent signal generated by a PCR assay reaction in real time, as well as the signal generated by internal positive control reagents, which are contained in the reagent beads.

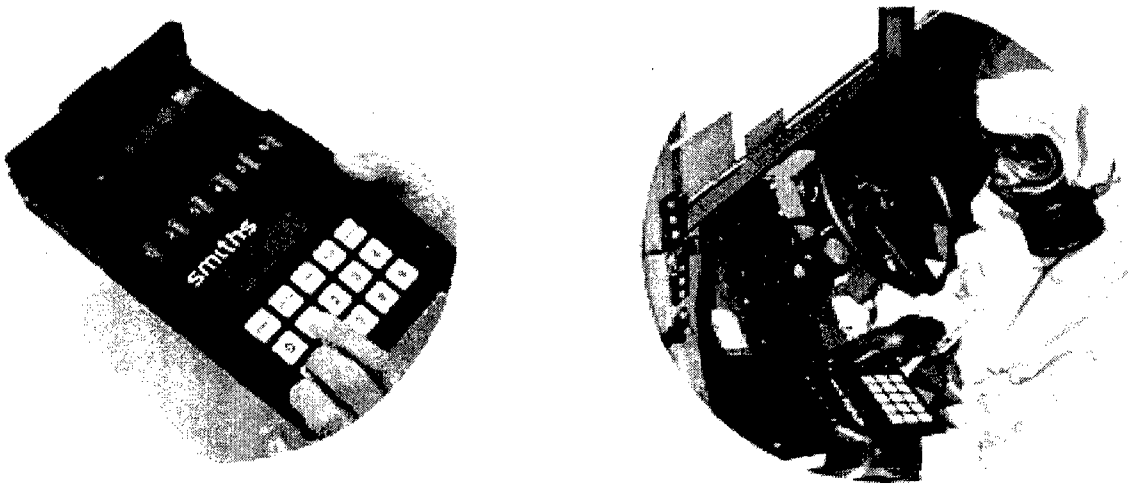

Figure 1. The Bio-Seeq® Instrument. Left: the lightshield is open, revealing the openings for 6 Bio-Seeq® tubes. Right: an illustration of the concept of operation. An operator, wearing protective equipment in a potentially hazardous environment, is about to conduct an assay.

The operator conducts an assay using a consumable sampling and reaction tube assembly (a consumable, Figure 2). The consumable is supplied in two assembled pieces. One piece consists of a buffer cup housing with a small handle that contains the buffer cup. Buffer is contained in the cup by a thin breakable plastic film. The second piece consists of a housing called a reagent base, which contains a hollow plunger tipped with a porous swab. Within the hollow plunger are three dried beads, which contain the assay reagents, and an inert mixing bead. A hole at the end of this piece leads into the attached clear plastic reaction tube.

To conduct an assay, the operator removes the two pieces from their packaging, and applies the swab-tipped end of the reagent base to the surface to be sampled. The swab tip is then inserted into the open end of the buffer cup holder and twisted. Threads on the buffer cup housing and the reagent base cause the swab tip to press against and break the film containing the buffer, which carries sample material through the porous tip and into the interior of the hollow plunger. By allowing the buffer to reside in the plunger for 90-s period, the buffer dissolves the reagent beads. The operator then shakes the device for 20 s causing the mixing bead to mix the sample, the buffer, and the assay reagents. The operator "whips" the assembled consumable to draw the aqueous reaction mixture into the reaction tube, which is then inserted into one of the six PCR modules in the Bio-Seeq® instrument. The operator then starts the program of PCR temperatures and durations appropriate to the particular assay reagents for that test.

4.2 Bacterial Strains, Culture Methods, and DNA Isolation.

The strains used in this study are listed in Table 1. All strains listed were obtained from an in-house culture collection at ECBC; many of the strains (for species other than *Y. pestis*) are available from ATCC (Manassas, VA). All strains used in this study were handled at the containment level appropriate to the organism (BSL-1, BSL-2, or BSL-3). DNA from several strains of *Y. pestis* was obtained from bacteria grown in the BL-3 laboratory, ECBC. *Y. pestis* strains were grown in liquid Luria broth (Difco) at 30 °C overnight with agitation. DNA was extracted from bacterial cells with QIAGEN DNeasy mini spin columns and reagents (QIAGEN, Valencia, CA), using the manufacturer's instructions for the isolation of total genomic DNA from gram-negative bacteria. DNA was quantified and tested for purity by measuring the absorbance spectrophotometrically at 260 nm and 280 nm. DNA from all isolations was dissolved and diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

The frequency of PCR assay targets was estimated to be one per genome copy. The number of copies of a *Y. pestis* genome per unit mass was calculated in the manner described below. An estimate of the genome size of *Y. pestis* (strain Kim) was obtained from the NCBI Microbial Genomes webpage.

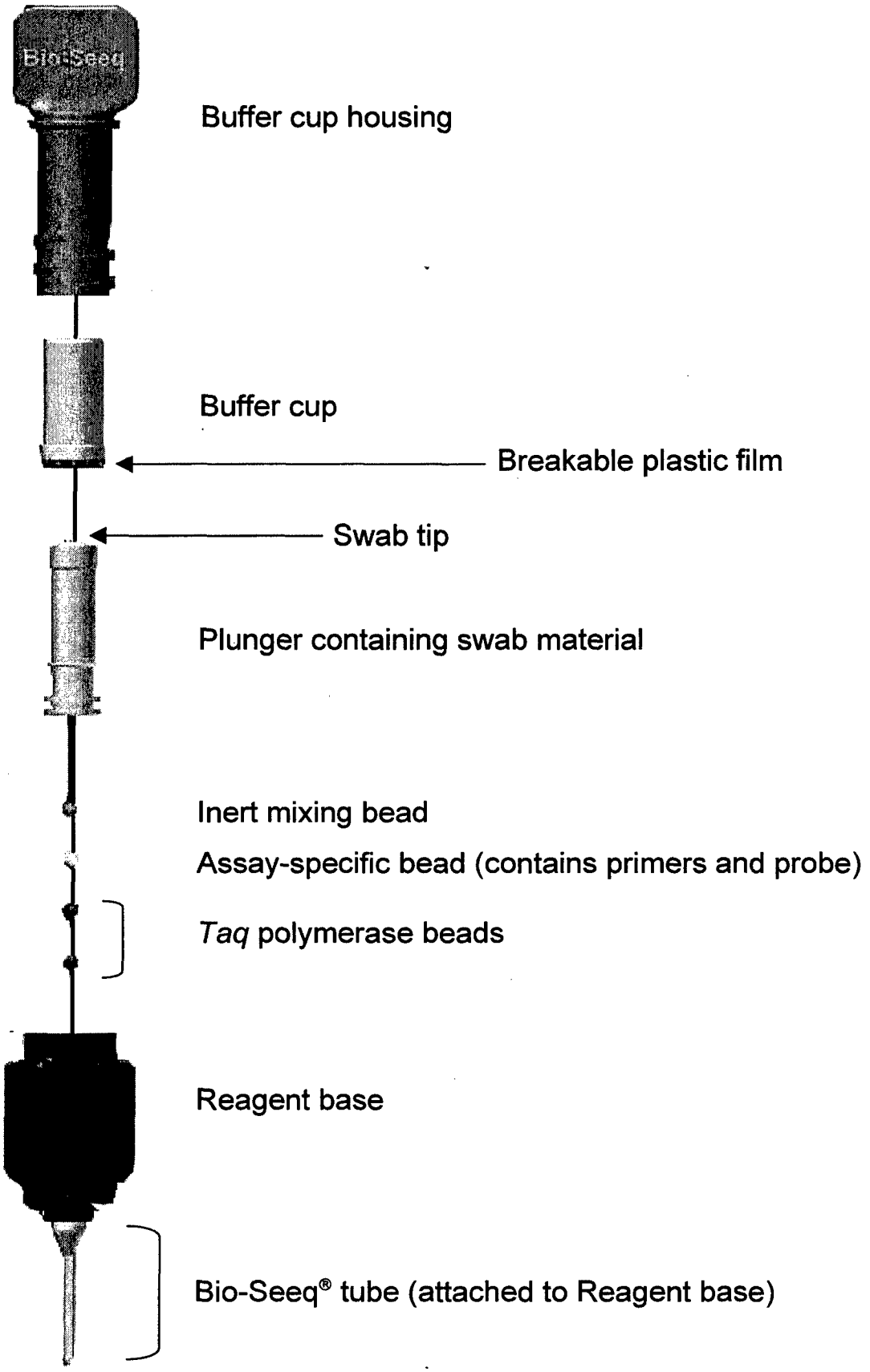

Figure 2. Consumable Sampling Device with Reaction Tube for the Bio-Seeq®

Table 1. Detection of Genomic DNA from *Y. pestis* Strains[a]

Yersinia pestis Strain	No. of Copies	No. Positive/Total[a]	Ct Values[b]
EV-76	1000	3/3	33, 33, 33
9910463	1000	3/3	33, 34, 36
9910265	1000	3/3	33, 33, 33
9910117	1000	3/3	33, 34, 34
9906414	1000	3/3	34, 33, 33
9808960	1000	3/3	33, 34, 33
9808780	1000	3/3	33, 33, 33
9808723	1000	3/3	34, 34, 34
9800419	1000	3/3	34, 33, 34
9709959	1000	3/3	33, 33, 33
97076011	1000	3/3	33, 34, 33
9168786	1000	3/3	34, 35, 34
9168781	1000	3/3	34, 34, 35
910810	1000	3/3	33, 33, 33
A294172	1000	3/3	34, 34, 34
16486	1000	3/3	33, 33, 33
A1122	1000	3/3	33, 34, 34
Amal	1000	3/3	32, 32, 33
Harbin	1000	3/3	33, 34, 34
Synthetic target	1000	6/7	35, 35, 35, 37, 38, 45
No template control	0	1/5	42[c]

[a] reactions run on either of two instruments. Data are combined from several experiments.
[b] N/A, not applicable. Values are reported only for positive assay results.
[c] False positive result due to faulty module.

To calculate the mass of 1000 genomes:

1. Calculate Molecular Weight (MW):

   = 4,627,242bp x 660 g/mole/bp = 3.05×10^9 g/mole of genomes

2. Calculate mass of 1000 genomes:

   3.05×10^9 g = 6.023×10^{23} genomes
   5.06×10^8 g = 10^{23} genomes (divide by 6.023)
   5.06×10^{-12} g = 10^3 genomes (divide by 10^{20})

Therefore, 1000 copies of a *Yersinia pestis* genome have a mass of approximately 5.06 picograms.

4.3 Choice of Interferents.

Interferents were chosen by the technical staff at SDE, in consultation with their marketing department. Four common materials were selected: coffee creamer (Domino Non-Dairy Creamer), baking powder (Rumford brand, Clabber Girl Inc., Terre Haute IN), wheat flour (America's Choice unbleached), and corn starch (Giant brand). Each material is found in most home kitchens and in many workplaces (especially coffee creamer). They may either be found on surfaces that have been contaminated with *Y. pestis* cells, or used by the perpetrator of a hoax, as it is widely believed that weaponized biological agents have the appearance of a white powder. All interferents were obtained by SDE staff at local supermarkets. Aliquots of each interferent were weighed and placed into 15 mL disposable plastic tubes before being supplied to the ECBC technical staff. Interferents were dissolved or suspended in nuclease-free, molecular biology grade water (Gibco BRL / Invitrogen, Carlsbad, CA) before use.

4.4 Assay Set-Up and Data Collection.

Assays were prepared in three ways:

1. To test the specificity and inclusivity of the probe and primer set using isolated total genomic DNA: for two reactions, a single dried assay specific bead containing the *Y. pestis* probe, primers, and internal control reagents was placed in a 1.5 mL microcentrifuge tube with two Ready-to-Go PCR reagent beads (containing *Taq* polymerase, dNTPs, and buffer) (Amersham Biosciences, Piscataway, NJ) and 48 μL nuclease-free water. When the reagent beads were dissolved, the mixture was divided into two 24 μL aliquots. One microliter of cells or DNA suspension was added to each reaction mixture (total volume 25 μL). Each reaction mixture was transferred to a Bio-Seeq® reaction tube, capped and covered with Parafilm, and gently centrifuged to draw the reaction mixture into the tube. Each tube was then placed into a separate PCR module in the Bio-Seeq® instrument and the thermocycling was started according to the manufacturer's instructions.

2. To test the limit of detection of the assay using viable cells as target, in an assembled consumable sampling device (a consumable; Figure 2): a single *Y. pestis* reagent bead and two Ready-to-Go PCR reagent beads were placed in a swab-tipped plunger with an inert mixing bead, and the plunger was inserted snuggly into the reagent base end of a consumable with a reaction tube affixed. Five microliters of a cell suspension (or DNA suspension or Tris buffer, in the control experiments) was spotted onto the surface of the swab tip. The consumable portion with the sample-bearing plunger, the reagents, and the reaction tube tip were then assembled with the portion containing the buffer cup. Twisting the two halves together broke the film on the buffer cup and allowed the sample and the reagent beads to be suspended in the buffer.

Assembled consumables were held inverted at room temperature for 90 s to allow the beads to dissolve, then shaken for 20 s to complete the dissolving and mix the reagents with the sample. The consumable was then "whipped" with a motion resembling that of shaking a Mercury thermometer to drive a portion of the reaction mixture into the reaction tube. The reagent tube tip of the consumable was then inserted into one of the six PCR modules in the Bio-Seeq® instrument.

3. To test the effect of interferent materials on the ability of the assay to detect *Y. pestis* cells: reagent beads were inserted into a swab-tipped plunger, the plunger was assembled with the reagent base end of a consumable, and the swab was spotted with target suspension as described above. However, interferent powders in suspension were not easily or accurately delivered by pipette to the swab tip, making necessary the following variation. One hundred thirty microliters of an aqueous suspension of an interferent was added to empty unsealed buffer cups, which was then gently assembled (horizontally) with the swap-tipped plunger (to which a sample target had already been added). The buffer cup holder was then placed around the buffer cup. The entire consumable was then assembled, interferent-containing buffer dissolved the reagents in the plunger, and the rest of the assay was performed as described above.

5. RESULTS AND DISCUSSION

5.1 Specificity of Reagents for *Yersinia pestis*.

The assay for *Y. pestis* detects the presence of a portion of a gene sequence on the chromosome. Total genomic DNA was obtained from our in-house DNA collection or prepared from each species and strain tested as described above. To determine whether the assay accurately identified *Y. pestis* strains, we diluted preparations of genomic DNA from several strains to a concentration of 1000 genome copies per microliter. One microliter of each DNA suspension was added to 24 μL of reaction mixture as described above, placed in a reaction tube and inserted into the instrument. As positive controls, we also assembled reactions containing 1000 copies of synthetic target DNA designed to match the assay reagents. The PCR thermocycler profile was run as pre-programmed by SDE.

When prepared as described above, the assay detected 1000 copies of genomic DNA from several strains of *Y. pestis*, as well as 1000 copies of the synthetic target (Table 1). All *Y. pestis* strains tested were detected.

The specificity of the assay was also determined by testing large amounts of total genomic DNA (100,000 copies per assay) from several other species of bacteria, as well as human DNA. No genomic DNA from the other species tested reacted positively in the *Y. pestis* assay (Table 2).

One of three replicate tests of the *Salmonella typhimurium* DNA produced a positive result on the Bio-Seeq. The weakness of the result (positive result called on the last PCR cycle, giving a C_T of 50) and the lack of response in two other assays lead us to believe that this result was anomalous and that *Salmonella typhimurium* does not cross-react with the assay.

5.2 Limits of Detection Using Purified Genomic DNA from *Y. pestis* Strain EV-76.

We determined the limit of detection for *Y. pestis* strain EV-76 DNA after noting that it reacted positively in the assay. *Y. pestis* strain EV-76 genomic DNA was diluted serially in TE buffer, and aliquots of each dilution were added to a reaction mixture as described above. The limit of detection for EV-76 genomes was between 125 and 62 copies of genomic DNA (Table 3).

5.3 Limits of Detection Using Cells of *Y. pestis* Strain EV-76.

Laboratory assays for the detection of *Y. pestis* strain EV-76 cells, using the reagent bead set (without the consumable), were prepared as described in Materials and Methods. A suspension of cells was serially diluted in sterile 10 mM Tris HCl, pH 7.4 (Sigma Co., St. Louis, MO), and aliquots containing cells were added to PCR reaction mixtures prepared in microcentrifuge tubes, then placed in Bio-Seeq® reaction tubes. The reagent bead set, prepared as described, allowed the detection of as few as 10-100 cells per assay (Table 4).

5.4 Limits of Detection of the YP Assay Using the Consumable Sampling Device.

To examine the sensitivity of the assay for *Y. pestis* strain EV-76 cells when the reagent bead set is incorporated into the consumable sampling device, we performed the assays at several cell concentrations, applying cells to the consumable by spotting five-1 μL aliquots onto the swab tip. The data (Table 5) indicate that the assay for *Y. pestis* strain EV-76 cells has a LOD between 10^3 and 5×10^3 cells per assay using the consumables. We noted, however, that the manner of assembling the consumable affected the sensitivity of the overall assay. In subsequent experiments, we found that a LOD of between 500 and 750 cells was achievable by assembling the buffer cup manually onto the plunger (Table 6).

Table 2. Specificity of YP Assay Reagents Tested Against Non-Target Genomic DNA

Source of Target DNA[a]	No. of Copies	No. Positive/Total[a]	Ct Values[b]
Yersinia enterocolitica	10^5	0/3	N/A
Yersinia rohdei	10^5	0/3	N/A
Fransicella tularensis Schu 4	10^5	0/3	N/A
Pantoea agglomerans[d]	10^5	0/3	N/A
Bacillus anthracis plasmid pXO1	10^5	0/3	N/A
Bacillus cereus ATCC 14579	10^5	0/3	N/A
Bordetella pertussis ATCC 9797	10^5	0/3	N/A
Campylobacter jejuni ATCC 33560	10^5	0/3	N/A
Clostridium perfringens	10^5	0/3	N/A
Escherichia coli ATCC 43895	10^5	0/3	N/A
Escherichia coli 0157:H7	10^5	0/3	N/A
Neisseria meningitides	10^5	0/3	N/A
Pseudomonas aeruginosa PAO1	10^5	0/3	N/A
Salmonella typhimurium LT2	10^5	1/3[c]	50
Staph. aureus ATCC 14458	10^5	0/3	N/A
Streptococcus pyogenes	10^5	0/3	N/A
Homo sapiens (placental)	10^5	0/3	N/A
synthetic target	1000	6/6	33, 36, 36, 36, 37, 46
No template control	0	0/3	N/A

[a] reactions run on either of two instruments. Data are combined from seven sets of experiments.
[b] N/A, not applicable. Values are reported only for positive assay results.
[c] False positive result due to faulty module.
[d] Species formerly known as *Erwinia herbicola*.

Table 3. Detection of *Y. pestis* Strain EV-76 Genomic DNA Using YP Reagent Beads[a]

No. of Genome Copies	No. Positive/Total[b]	Ct Values[c]
10000	4/4	30, 30, 30, 30
1000	5/5	28, 33, 33, 33, 33
750	5/5	31, 33, 33, 33, 34
500	5/5	29. 34, 35, 35, 35
250	5/5	34, 35, 35, 36, 36
125	6/6	34, 36, 36, 36, 45
62	2/5	30, 50
50	3/5	31, 40, 47
40	4/5	42, 47, 50, 50
30	1/5	44
20	0/3	N/A
0 (buffer alone)	0/4	N/A
Synthetic target (1000 copies)	2/2	36, 36

[a] Reaction mixtures prepared in a microcentrifuge tube and placed directly into a Bio-Seeq® reaction tube, without passing through the complete, assembled consumable sampling device. Results are combined from two experiments.
[b] reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.

Table 4. Detection of *Y. pestis* Strain EV-76 Cells Using YP Reagent Beads[a]

No. of Colony-Forming Units	No. Positive/Total[b]	Ct Values[c]
5×10^7	5/5	18, 18, 19, 20, 31
10^6	11/11	2, 18, 21, 5@22, 2@23, 24
10^5	11/11	22, 2@25, 7@26, 27
10^4	11/11	25, 27, 2@28, 5@29, 2@30
5×10^3	11/11	26, 2@29, 6@30, 2@31
10^3	11/11	29, 6@32, 3@33, 34
10^2	11/11	19, 30, 31, 34, 5@35, 36, 38
10^1	10/11	4@37, 3@38, 39, 45, 46
1 cfu	0/11	N/A
0.1 cfu[d]	0/11	N/A
Synthetic target (1000 copies)	10/10	32, 2@35, 6@36, 37

[a] Reaction mixtures prepared in a microcentrifuge tube and placed directly into a Bio-Seeq® reaction tube, without passing through the complete, assembled consumable sampling device. Results are combined from four experiments.
[b] reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.
[d] A further 10-fold dilution of the previous sample.

Table 5. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device

No. of Colony-Forming Units	No. Positive/Total[a]	Ct Values[b]
10^6	3/3	22, 30, 32
10^5	3/3	29, 30, 32
10^4	3/3	36, 39, 47
$5x10^3$	3/3	38, 38, 43
10^3	2/3	41, 49
10^2	1/3	48
10^1	1/3	21
1 cfu	0/3	N/A
0.1 cfu[c]	0/3	N/A

[a] reactions run on either of two instruments.
[b] N/A, not applicable. Values are reported only for positive assay results.
[c] A further 10-fold dilution of the previous sample.

Table 6. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device, Modified Method of Assembly

No. of Colony-Forming Units	No. Positive/Total[a]	Ct Values[b]
$5x10^3$	9/9	35, 28, 36, 20, 36, 34
$3x10^3$	6/6	36, 36, 18, 34, 40, 41
10^3	6/6	36, 37, 38, 34, 39, 35
750	6/6	36, 39, 18, 32, 38, 30
500	5/6	38, 23, 22, 37, 22

[a] reactions run on either of two instruments.
[b] N/A, not applicable. Values are reported only for positive assay results.

5.5 Effects of Interferents in Laboratory Tests of the YP Assay Using the Consumable Sampling Device.

Two concentrations of each inhibitor were used to test the effect of each on the ability of the assay to detect cells of *Y. pestis* strain EV-76. The SDE staff determined the concentrations to be tested based upon work done at the SDE laboratories. The number of cells to be tested were 5 x 10^3 cfu per assay (the lowest consistently detected number of cells when assay were performed using the consumable sampling device (Table 5), and 2-fold and 20-fold excesses of that amount. All inhibitors were dissolved/suspended in nuclease-free water. All cell suspensions were diluted in 10 mM Tris HCl, pH 7.4. Tris diluent was used in place of cells in all of the negative controls.

Cornstarch had no effect on the performance of the assay at either amount added (Table 7). Coffee creamer, at the lower concentration (10 mg per assay) also had little effect on the performance of the assay (Table 8). However, the higher concentration of coffee creamer tested (15 mg per assay) did significantly degrade the ability of the assay to consistently detect cells of *Y. pestis* strain EV-76.

Table 7. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device in the Presence of Cornstarch

Amount Per Assay[a]	No. of Cells Added	No. Positive/Total[b]	Ct Values[c]
5 mg	10^5	3/3	32, 35, 37
	10^4	3/3	34, 34, 40
	5×10^3	3/3	34, 35, 40
7.5 mg	10^5	3/3	31, 32, 36
	10^4	3/3	33, 35, 38
	5×10^3	3/3	36, 37, 37
	Positive control (10^5 cells)	2/2	30, 32
	NTC	0/2	N/A

[a] Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b] Reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.

Table 8. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device in the Presence of Coffee Creamer

Amount Per Assay[a]	No. of Cells Added	No. Positive/Total[b]	Ct Values[c]
10 mg	10^5	3/3	27, 31, 35
	10^4	3/3	23, 32, 34
	5×10^3	3/3	31, 33, 36
15 mg	10^5	2/3	26, 41
	10^4	3/3	32, 34, 39
	5×10^3	1/3	39
	Positive control (10^5 cells)	2/2	29, 35
	NTC	0/2	N/A

[a] Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b] Reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.

As observed in the previous studies,[5,6] an examination of the consumable suggests that the amounts of coffee creamer and cornstarch needed to partially inhibit the performance of the assay are far in excess of the amounts that would adhere to the swab tip when sampling a surface. We conclude from these results that neither coffee creamer nor cornstarch significantly inhibits the assay for *Y. pestis* in amounts likely to be introduced into the interior of the consumable.

The presence of flour significantly degraded the efficacy of the YP assay at both concentrations tested (0.2 mg or 0.3 mg per assay) (Table 9).

Table 9. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device in the Presence of Wheat Flour

Amount Per Assay[a]	No. of Spores Cells Added	No. Positive/Total[b]	Ct Values[c]
0.2 mg	10^5	3/3	33, 33, 34
	10^4	2/3	35, 38
	5×10^3	2/3	36, 42
0.3 mg	10^5	0/3	N/A
	10^4	3/3	35, 37, 47
	5×10^3	2/3	23, 37
	Positive control (10^5 cells)	2/2	32, 33
	NTC	½	24[d]

[a] Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b] Reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.
[d] Inspection of data (not shown) reveals result consistent with air bubble in sample.

The concentrations of baking powder tested in this study did not inhibit the performance of the *Y. pestis* assay (Table 10). Conversely, the minimum inhibitory concentration of baking powder observed in the study of the *B. anthracis* assay was between 0.025 and 0.0025 mg per assay when 10^4 spores were detected.[6] In this study, we tested only the two highest concentrations, 0.050 and 0.075 mg baking powder per assay. Additional control experiments (no interferent added), in which 10^5, 10^4, or 5×10^3 cells were added, all gave positive results (data not shown).

Table 10. Detection of *Y. pestis* Strain EV-76 Cells Applied to the Consumable Sampling Device in the Presence of Baking Powder

Amount Per Assay[a]	No. of Spores Cells Added	No. Positive/Total[b]	Ct Values[c]
0.05 mg	10^5	3/3	29, 31, 37
	10^4	3/3	33, 41, 41
	5×10^3	3/3	35, 35, 36
0.075 mg	10^5	3/3	31, 31, 36
	10^4	3/3	34, 34, 38
	5×10^3	3/3	37, 36, 43
	Positive control (10^4 cells)	2/2	35, 41
	NTC	0/2	N/A

[a] Total amount of interferent introduced into the consumable buffer cup in 130 µL of water. NTC, no-template control (negative control).
[b] Reactions run on either of two instruments.
[c] N/A, not applicable. Values are reported only for positive assay results.

6. CONCLUSIONS

The Bio-Seeq® instrument and reagents for *Yersinia pestis* assays correctly identified all 19 *Y. pestis* strains listed in Table 1 and did not react with 15 other species and strains of bacteria, nor human placental DNA (Table 2). Under laboratory conditions, prepared in microcentrifuge tubes, the sensitivity of the assay was observed to be as low as 62-125 copies of the genome (Table 3), which was similar to the LOD we determined for intact cells (as low as 10-100 cells per assay) (Table 4). The observed LOD while using the consumable was 1000-5000 cells (Table 5). However, a modified method of assembly allowed us to detect between 500 and 750 cells (Table 6).

The addition of cornstarch (Table 7) or coffee creamer (Table 8) had little effect on the performance of the assay under the conditions tested. However, flour (Table 9) adversely affected assay performance, reducing the sensitivity approximately 20-fold when present at 0.2 mg per assay.

The presence of baking powder was not observed to inhibit the performance of the assay in this study. However, given the very low concentration used and the results observed in studies of the assays for *B. anthracis* and *F. tularensis*, we anticipate that concentrations of baking powder not much greater than those used in this study might have a significant impact on the performance of the *Y. pestis* assay. However, we also must point out that the consumable sampling devices were handled differently during the testing of baking powder as an inhibitor. Open buffer cups containing 130 µL of buffer were removed from buffer cup housings and placed

manually on the plunger. Buffer cups were then snapped into place before screwing on the buffer cup housing. This method allowed a greater amount of liquid to enter the inner chamber containing the assay beads. After the buffer cup housing was snapped into place (screwed on), the original protocol was followed. We also observed in additional limit-of-detection experiments that this method of assembling the consumable conferred greater sensitivity on the assay (Table 6). We have been informed by technical staff at Smith Detection - Edgewood that modifications to the design of the consumable sampling device have been made that result in this improved form of assembly and will result in improved assay sensitivity.

We also note that no sample preparation or DNA clean-up steps were involved in the assays performed on intact cells in this study, although the heat of thermocycling is likely to degrade the integrity of cells of gram-negative bacteria. Sample processing steps including cell lysis (resulting in recovery of most of the sample DNA in pure form) should increase the sensitivity of the assay, as well as remove many of the problems caused by the presence of interferents.

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